C terminal (pARC8 plasmid) Incremental Truncation Protocol

Incremental truncation protocol adapted from protocol in from Methods in Ezymology, "Construction or Protein Fragment Complementation Libraries Using Incremental Truncation" Paschon, D. and Ostermeier, M.

A. Preparation of Plasmid DNA for Truncation

C terminal fragment with pARC8 or pDIM-C8

1. Prepare digestion mixtures
   - 10µg of pARC8 or pDIM-C8 with gene insert
   - 20µL of 10x NEB buffer 1
   - 2µl of 100x bovine serum albumin (BSA)
   - 7.5µl of SacI (150 units)
   - 10 µl of XhoI (200 units)
   - and water to 200µl
2. Incubate digestions at 37°C for 1.5-2hrs
3. Incubate at 65°C for 20 min to inactivate enzymes
4. Add 5x volume of digestions of QIAquick buffer PB
5. Follow QIAquick protocol
6. Elute DNA from column with 50µl of QIAquick buffer EB

B. Construction of Individual Truncation Libraries

1. Equilibrate 250µl QIAquick buffer PB at room temperature in 1.5ml tube (tube A)
2. Into a 0.5ml tube (tube B) add
   - 4µg digested DNA
   - 12µl of 10x ExoIII buffer
   - 8.4ul amount of 1 M NaCl (final conc of 70mM*)
   - Water to 120µl
   - *Conc of NaCl determines rate according to following equation

     $$\text{Rate(bp/min)} = 48 \times 10^{-0.00644[\text{NaCl}]}$$

3. Equilibrate tube B in a minifridge or thermocycler at 22°C
4. At time t=0, add 400 units (4µl) of Exo III to tube B and mix immediately
5. Remove 0.6µl samples from tube B every 20 s and add to tube A, mix tube A well. Leave tube B open during sampling to avoid temperature change due to handling.
6. After all samples are taken estimate volume remaining in Tube A and add 5x QIAquick buffer PB.
7. Follow QIAquick protocol
8. In final step elute with 44µl buffer EB
9. Add 5µl of 10x mung bean nuclease buffer and 3µl (3units) of mung bean nuclease and incubate tube at 30°C for 30min.
10. Add 250 µl of QIAquick buffer PB
11. Follow QIAquick protocol
12. Elute truncated DNA form QIAquick column with 90µl
13. Equilibriate pARC8 at 37°C

B Continued

14. Add
    - 11µl NEB Buffer 2
    - 0.5µl Klenow (5,000U/ml)
    - 10µl dNTPs (0.125mM each)

    Incubate at 37°C for 5minutes
15. Deactivate Klenow @72°C for 20 minutes
16. Cool to room temperature and add
    - 0.4ml ligase mix
        - 320µl DI H_2O
        - 40µl 10x T4 DNA Ligase buffer
        - 40µl 50% PEG 8000
        - 3µl T4 DNA ligase (18 Weise Units)
17. Incubate at room temperature for ≥12hrs
18. Concentrate by ethanol precipitation
    - To 250µl of ligation mix add
        - 125µl 7.5M ammonium acetate
        - 750µl of 100% ethanol (at -20°C)
        - 2µl Pellet Paint (Novagen)
    - Incubate on ice for 30 min
    - Centrifuge at 10,000g at 4°C for 10 min
    - Wash DNA pellet with 750µl of 70% ethanol (at -20°C)
    - Spin an additional 2 min and thoroughly remove liquid by pipetting
    - Air dry DNA pellet for 10 min and resuspend in 15µl H_2O.
19. Electroporate ≤ 5µl of DNA into 40µl of electrocompetent DH5α-E E. Coli cells (Bio-Rad Gene Pulser II set to 25µF capacitance, 200 Ω resistance, and 2.0 kV). Allow cells to recover in 1ml of SOC media while shaking at 200rpm at 37°C.
20. Plate 1µl of cells on small plate (LB with Agar) with 50µg/ml chloramphenicol (Cm). Plate remaining mixture on a large LB plate (e.g., 245 x 245-mm bioassay dish) with Cm. Colonies on small plate used to determine # of transformants (total transformants = number of colonies on small plate x 1000) and will be used for evaluating libraries.
21. Incubate plates overnight at 37°C

C. Recovering and Storing Individual Truncation Libraries

1. Recover cells from 245 x245-mm bioassay dish by adding 2 x 15ml storage media to top of plate and scraping off cells using a cell spreader and then pipette cells into a 50ml polypropylene centrifuge tube.
    - For storage media add
        - 18ml LB
        - 9 ml 50% glycerol
        - 3ml 20% (w/v) glucose
2. Spin cells in a centrifuge at 5000rpm at 4°C for 10 min
3. Decant supernatant and add 2ml storage media
4. Resuspend pelleted cells by gentle shaking
5. Store 4 x 200µl aliquots in 1.5-ml tubes at -80°C

6. Pellet remaining cells in centrifuge at 5000rpm at 4°C and follow protocol for QIAGEN HiSpeed plasmid midiprep kit to recover library in DNA form.

D. Evaluating the Individual Truncation Libraries

1. Combine
   - 70µl 10x Taq buffer (without $MgCl_2$)
   - 14µl dNTPs (10mM each)
   - 42µl 25mM $MgCl_2$
   - 42µl of 10µM forward primer (Czip-for)
   - 42µl of 10µM reverse primer (pARC8-rev)
   - 4µl Taq DNA Polymerase (20 units Promega)
   - Fill to 700µL with H_2O
2. Add 25 µl of PCR mixture per tube
3. Transfer one colony per tube from 1-µl plate using a sterile pipette tip. Also perform a control PCR on original plasmid using same primers as above.
4. Perform PCR for 30 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C.
   - 1 94˚C-5 min
   - 2 94˚C-1 min
   - 3 56˚C-1 min
   - 4 72˚C-1 min
   - Repeat steps 2-4 30 times
   - 5 72˚C-10min
   - 6 4˚C - hold

5. Analyze PCR reactions by Agarose gel electrophoresis
   - Make 125ml of 1 % Ultrapure agarose gel
   - Dilute PCR samples to 5µl PCR and 5µl H_2O and add 2µl of 6x Loading Dye.
   - Run Gel for 40 minutes at 100V

If library is biased toward too little truncation, use a lower concentration of NaCl. If library is biased toward too much truncation, use higher concentration of NaCl.